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Date: February 29, 2008
Number of pages including this one: - 2-

TO:
Examiner **Mrs. Sandra L. Wegert**
Firm: U.S.P.T.O.
Fax: 5~~2~~273-0895
571

FROM:
Name: Ronald S. Kosie
Direct line: (514) 397-6942
E-mail: rsk@bcf.ca
Ref. No.: 13545-006

Operator: Janique Forget
Telephone: (514) 397-8500 / 397-6699
Extension: 6906

COMMENTS:

Re : U.S. Patent Application No. 10/718,598
Filed on November 24, 2003
Title : METHOD FOR MAKING AND DELIVERING RHO-ANTAGONIST TISSUE ADHESIVE FORMULATIONS TO THE INJURED MAMMALIAN CENTRAL AND PERIPHERAL NERVOUS SYSTEMS AND USES THEREOF
O/Ref. : 13545-006

Dear Mrs. Wegert

The present relates to our telephone conversation of today's date.

Please find enclosed a copy of the acknowledgement postcard date stamped by the U.S.P.T.O as evidence of the submission and receipt by the U.S.P.T.O of an Information Disclosure Statement on October 30, 2007.

With best regards,

Ronald S. Kosie
Reg. No. 28,814
Telephone: (514) 397-6942
Fax: (514) 397-8515

BCF LLP, 1100 René-Lévesque Blvd. West, 25th Floor, Montréal, Québec CANADA H3B 5C9
Telephone: (514) 397-8500 Fax: (514) 397-8515

PATENT PROVIDERS

DUE DATE: UPON RECEIPT

ATTORNEY DOCKET NO.: 13545-006 JF/cd

ENCLOSURES:

1. cover letter to Quality Patent;
2. Cover letter to the USPTO and Filing particulars;
3. Forms PTO/SB/08A and PTO/SB/08B;
4. Copies of listed non-patents document;
5. Post card to U.S.P.T.O.

STAMP, DATE AND RETURN: October 30, 2007,

OK TO ENTER
SLW 2/29/08

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

U.S. Patent Application No.	:	10/718,598
Filed on	:	November 24th, 2003
Title	:	METHOD FOR MAKING AND DELIVERING RHO-ANTAGONIST TISSUE ADHESIVE FORMULATIONS TO THE INJURED MAMMALIAN CENTRAL AND PERIPHERAL NERVOUS SYSTEMS AND USES THEREOF
Applicant	:	Lisa McKerracher
Examiner:	:	Sandra L. Wegert
File No.	:	13545-006 (formerly 06447-011) JFO / cd

Montreal, Quebec, Canada
October 30th, 2007

MAIL STOP AMENDMENT
Commissioner for Patents
U.S. Patent and Trademark Office
P.O.BOX 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

The Applicant hereby submits on forms PTO/SB/08A and PTO/SB/08B, the listing of documents known to the Applicant in order to comply with Applicant's duty of disclosure.

The above mentioned patent application is a divisional of U.S. Ser. No. 09/725,906 now U.S. Patent No. 7,141,428 (the earlier application). The references listed on the attached forms were submitted to and/or cited by the Patent Office during prosecution of the earlier application. In accordance with 37 C.F.R. 1.98(d), the earlier application has been properly identified in the attached information disclosure statement and is relied on for an earlier effective filing date under 35 U.S.C. 120. The Applicant believes the information disclosure statement submitted in the earlier application complies with 37 C.F.R. 1.98 paragraphs(a) to (c). As such, copies of references provided in the earlier application are not provided herein. If the Examiner finds it otherwise, the Applicant will gladly provide copies of these references. Copies of any listed U.S. patents or U.S. patent application publication can also be provided upon request. Consideration of the references submitted by Applicant is respectfully respected.

This statement is being filed after a first Office Action on the merits, but before receipt of a final Office Action or a Notice of Allowance. There is a late submission fee of $180 under 37 C.F.R.

1.17(p). The United States Patent and Trademark Office is hereby authorized to charge the late submission fee of $180 to our deposit account no.02-3980.

If any fees whatsoever are due with respect to the present application, the United States Patent and Trademark Office is hereby authorized to charge any such fee to our deposit account no.02-3980

Respectfully submitted,

By:

Gaétan Prince
Patent Agent Reg. No. 33107
(514) 397-6725

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 10

Complete if Known	
Application Number	10/718,598
Filing Date	November 24th, 2003
First Named Inventor	McKERRACHER Lisa
Art Unit	1647
Examiner Name	Sandra L. Wegert
Attorney Docket Number	13545-006

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US-	—	—	—
SLW		US- 4359049	11-16-1982	Redl et al.	
		US- 4874368	10-17-1989	Miller et al.	
		US- 4978336	12-18-1990	Capozzi et al.	
		US- 5900408	05-04-1999	Block et al.	
		US- 5922356	07-13-1999	Koseki et al.	
		US- 5945115	08-31-1999	Dunn et al.	
		US- 5989215	11-23-1999	Delmotte et al.	
		US- 6036955	03-14-2000	Thorper et al.	
		US- 6047861	04-11-2000	Vidal et al.	
		US- 6117425	09-12-2000	MacPhee et al.	
		US- 6121422	09-19-2000	Zimmerman et al.	
		US- 6124273	09-26-2000	Drohan et al.	
		US- 6218410	04-17-2001	Uehata et al.	
		US- 4897834	05-03-1991	Muro et al.	
		US- 7141428	28-11-2006	Université de Montréal	
		US-			
		US-			
		US-			

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
SLW		CA-2300878	02-25-1999	Strittmatter		
SLW		EP-0956865	19/02/1998	Yoshitomi Phar Ind.		
SLW		WO98/06433	19/02/1998	Yoshitomi Phar Ind.		

Examiner Signature	Sandra Wegert	Date Considered	2/29/08

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This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: **Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Sheet 2 of 10

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Application Number	10/718,598
Filing Date	November 24th, 2003
First Named Inventor	McKERRACHER Lisa
Art Unit	1647
Examiner Name	Sandra L. Wegert
Attorney Docket Number	13545-006

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
SLW		Masuda-Nakagawa, L., et al, 1993, PNAS, 90: 4966-4970.	
		Ramon-Cueto et al. Neuron 25:425-435 (2000)	
		Hauser, et al, 1993, J. Bacteriol., 175(22): 7260-7268.	
		Moriishi, et al, 1993, Infection and Immunity, 61(12): 5309-5314.	
		Omelchenko, et al, 2003, PNAS, 100(19): 10788-1079.	
		Spronk, et al, 2004, Thrombosis J., 2: 12-21.	
		Ten Berg, et al, 2001, Curr. Control. Trials Cardiovasc. Med., 2: 129-140.	
		Ishizaki, et al, 2000, Mol. Pharmacol., 57: 976-983.	
		Winton, et al, 2002, J. Biol. Chem., 277(36): 32820-32829.	

Examiner Signature	Sandra Wegert	Date Considered	2/29/08

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Sheet 3 of 10

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SLW		Taniguchi-Sidle, et al, 1992, J. Biol. Chem, 287(1): 635-643.	
		Itoh, et al, 1999, Nature Medicine, 5(2): 221-225.	
		Blazso, et al, 2004, Phytother. Res., 18(7): 579-581.	
		Itano, et al, 2002, Proc. Natl. Acad. Sci., 99(6): 3609-3614.	
		Saito, et al, 1995, FEBS, 371: 105-109.	
		Boston life sciences, Sep. 6, 2000 Press release.	
		Aguayo , et al. , J. Exp. Biol.95:231-40 (1981).	
		Schwab et al. Annu. Rev. Neurosci. 16:565-595 (1993).	
		Schnell and Schwab Nature 343:269-272 (1990).	
		Weibel, et al. Brain Res 642:259-266 (1994).	

Examiner Signature	Sandra Wegert	Date Considered	2/29/08

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Sheet 4 of 10

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Application Number	10/718,598
Filing Date	November 24th, 2003
First Named Inventor	McKERRACHER Lisa
Art Unit	1647
Examiner Name	Sandra L. Wegert
Attorney Docket Number	13545-006

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SLW		Ramer, et al. Nature 403:312-316 (2000).	
		Liu, et al. J. Neurosci 19:4370-87 (1999).	
		Blesh, et al., J. Neurosci 19:3556-66 (1999).	
		Schnell, et al., Nature 367:170-173 (1994).	
		Neuman, Neuron 2383-91 (1999).	
		Cai, et al., Neuron 22:89-101 (1999).	
		Lehmann, et al. J. Neurosci 19:7537-7547 (1999).	
		Li, et al., J. Neurosci res. 46:404-414 (1996).	
		Fan, et al., J. Cell Biol. 121:867-878 (1993).	
		Tigyi, et al., Journal of Neurochemistry 66:537-548 (1996).	

Examiner Signature	Sandra Wegert	Date Considered	2/29/08

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Sheet 5 of 10

	Complete if Known
Application Number	10/718,598
Filing Date	November 24th, 2003
First Named Inventor	McKERRACHER Lisa
Art Unit	1647
Examiner Name	Sandra L. Wegert
Attorney Docket Number	13545-006

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SLW		Kuhn, et al. J. Neurosci 19:1965-1975 (1999).	
		Jin and Strittmatter, J. Neurosci 17:6256-6263 (1997).	
		Zheng and Li, J. Biol. Chem. 272:4671-4679 (1999).	
		van Leeuwen, et al. J. Cell Biol. 139:797-807 (1997).	
		Nobes and Hall, Cell 1995.81:53-62 (1995).	
		Laudanna, et al., Science 271:981-983 (1996).	
		Hannigan, et al., Nature 379:91-96 (1996).	
		Kuhn, et al., J. Neurobiol. 37:524-540 (1998).	
		Hall, Ann. Rev. Cell. Biol. 10:31-54 (1996).	
		Kozma, et al., Molec. Cell. Biol. 17:1201-1211 (1997).	

Examiner Signature	Sandra Wegert	Date Considered	2/29/08

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Sheet 6 of 10

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Application Number	10/718,598
Filing Date	November 24th, 2003
First Named Inventor	McKERRACHER Lisa
Art Unit	1647
Examiner Name	Sandra L. Wegert
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SLW		Albertinazzi, et al. J. Cell. Biol. 142:815-825 (1998).	
		Huthchens, et al. Molec. Biol. Cell 8:481-500 (1997).	
		Daniels, et al., EMBO Journal 17:754-764 (1998).	
		Sebok, et al. J. Neurochem 73:949-960 (1999).	
		Lang, et al., EMBO Journal 15:510-519 (1996).	
		Dong, et al., J. Biol. Chem 273:22554-22562 (1998).	
		Renaudin, et al., J, Neurosci Res. 55:458-471 (1998).	
		Dillon and Feigh, Methods in Enzymology: Small GTPases and their regulators Part. B.256:174-184 (1995).	
		Kimura and Schubert, Journal of Cell Biology 116:777-783 (1992).	
		Keino-Masu, et al., Cell. 87:175-185 (1996).	

Examiner Signature	Sandra Wegert	Date Considered	2/29/08

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Sheet 7 of 10

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Application Number	10/718,598
Filing Date	November 24th, 2003
First Named Inventor	McKERRACHER Lisa
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Examiner Name	Sandra L. Wegert
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SLW		Matsui, et al., EMBO J. 15:2208-2216 (1996).	
		Matsui, et al., J. Cell Biol. 140:647-657 (1998).	
		Ishizaki, FEBS Lett. 404:118-124 (1997).	
		Vaheri, et al., Curr. Opin. Cell. Biol. 9:659-666 (1997).	
		Goslin, et al., J. Cell Biol. 109:1621-1631 (1989).	
		Hirose, et al., J. Cell Biol. 141:1625-1636 (1998).	
		Bito, Neuron 26:431-441 (2000).	
		Ishizali, et al., Molecular Pharacology 57:976-983 3 (2000).	
		Uehata, et al., Nature 389:990-994 (1997).	
↓		Somlyo, Nature 389:908-911 (1997).	

Examiner Signature	Sandra Wegert	Date Considered	2/29/08

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		Guest, J. Neurosci Res. 50:888-905 (1997).	
		Verge, et al., Journal of Neuroscience 15:2081-2096 (1995).	
		Cheng, et al., Science, 273:510-513 (1996).	
		Joosten, J. Neurosci Res. 41:481-490 (1995).	
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 9 of 10

	Complete if Known
Application Number	10/718,598
Filing Date	November 24th, 2003
First Named Inventor	McKERRACHER Lisa
Art Unit	1647
Examiner Name	Sandra L. Wegert
Attorney Docket Number	13545-006

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
SLW		Tuszynsli, et al., Cell Transplant 7:187-96 (1998).	
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		Li, et al. Science 277:2000-2002 (1997).	
↓		Ramon-Cueto, et al., J. Neurosci 18:3803-15 (1998).	

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Y		Methods in Enzymology, vol. 256, Part B., Eds.: W.E. Balch, C.H. Der, and A. Hall. Academic Press, 1995	

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Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1632–1636, March 1994
Neurobiology

Effects of ocular injury and administration of brain-derived neurotrophic factor on survival and regrowth of axotomized retinal ganglion cells

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Communicated by Walle J. H. Nauta, November 16, 1993 (received for review June 30, 1993)

ABSTRACT Optic nerve transection in adult rats results in the death of ~50% of the axotomized retinal ganglion cells (RGCs) by 1 week and nearly 90% by 2 weeks after injury. The capacity of brain-derived neurotrophic factor (BDNF) to prevent this early, severe loss of RGCs was investigated *in vivo* by intravitreal injections of BDNF [5 μg in 5 μl of bovine serum albumin/phosphate-buffered saline (BSA/PBS)] or vehicle (5 μl of BSA/PBS). Using quantitative anatomical techniques, we show that (*i*) all RGCs survived 1 week after a single injection of BDNF at the time of axotomy, (*ii*) RGC densities decreased in the BDNF-treated retinas by 2 weeks but remained significantly greater than in the untreated controls, (*iii*) An enhanced RGC survival was obtained with single injections of BDNF from 6 days before to 5 days after axotomy, (*iv*) Repeated injections resulted in greater numbers of surviving RGCs, an effect that declined to undetectable levels by 6 weeks, (*v*) There were indications for an endogenous local source of trophic support whose expression was triggered by ocular injury, particularly to the anterior part of the eye, (*vi*) With multiple BDNF injections, there was profuse axonal sprouting around the optic disc. This remarkable intraretinal growth was not, however, reflected in increased RGC innervation of the peripheral nerve grafts, which are known to facilitate regeneration when used as optic nerve substitutes.

Axonal injury in the central nervous system (CNS) of adult mammals often results in neuronal death. In rats, for example, 80–90% of the retinal ganglion cells (RGCs) are lost within 2 weeks of optic nerve (ON) transection near the eye (1). These and other neurons axotomized near their somata are presumed to die because they are deprived of the trophic support that is normally provided by their distant targets and by the nonneuronal cells that surround their axons.

Some of the axotomized RGCs that survive ON section regrow their axons when the CNS glial environment in the ON is changed by grafting a segment of sciatic nerve. Under such experimental conditions, RGC axons can extend several centimeters along the peripheral nerve (PN) graft and form new functional synapses in the superior colliculus (SC) (2). While the reestablishment of such connections within the SC appears to prevent further RGC loss (3), few axons reach this target, presumably because so many RGCs die soon after axotomy. Thus, timely administration of specific molecules capable of enhancing the survival of these injured neurons could have important effects on the overall regenerative response of injured RGCs.

Several lines of evidence suggest that brain-derived neurotrophic factor (BDNF) is a specific trophic molecule for RGCs. The survival of RGCs *in vitro* is enhanced by BDNF (4, 5) and BDNF mRNAs are present in the retina (6), ON (M. J. Berkelaar, T. N. Jelsma, G.M.B., and A.J.A., unpublished observations), and SC (6, 7). Furthermore, RGCs express the mRNA for trkB (8), the functional receptor for BDNF (9). Here we have documented quantitatively in adult rats the effects of early intravitreal administration of human recombinant BDNF on the survival of axotomized RGCs and investigated the regrowth of RGC axons in both the retina and in grafted segments of PN used as ON substitutes.

MATERIALS AND METHODS

All surgical procedures, including intraocular injections, were performed in female Sprague–Dawley rats (180–200 g) under general anesthesia (7% chloral hydrate; 42 mg per g of body weight, i.p.) and in accordance with the principles outlined (10).

RGC Labeling. RGCs were retrogradely labeled with Fluorogold (Fluorochrome, Englewood, CO; 2% in 0.9% NaCl containing 10% dimethyl sulfoxide) applied to the surface of both SC, as described for 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (diI) (11, 12). For the experiments in which a correlation between RGC survival and axonal regrowth was investigated (as described below), horseradish peroxidase (HRP; Boehringer Mannheim) was applied to the distal tip of the PN graft, ~1 cm from the eye, 6 weeks after the graft was attached to the ocular stump of the ON (13).

ON Transection. One week after Fluorogold application, the left ON was transected 0.5 mm from the eye (1). In some animals, a segment of autologous sciatic nerve was attached to the ocular stump of the transected ON to test the capacity of the RGCs to regenerate and extend their axons (13).

Injection Procedure. Anesthetized animals received single injections 3 or 6 days before or 0–10 days after ON transection. Multiple injections were given on postoperative days 0, 3, 7, and 10 for the animals without PN grafts and on days 0, 3, and 7 for the animals with PN grafts. Intraocular injections were made with a 10-μl Hamilton syringe fitted with a 26-gauge needle whose tip was inserted into the vitreous space by an anterior or posterior approach. For the anterior approach, a drop of 2% lidocaine (Xylocaine) was applied to the conjunctiva, and the needle was inserted through the cornea–sclera junction and advanced into the vitreous chamber, avoiding direct contact with the retina. By this approach, the needle usually pierced the margins of the iris and could damage the surface of the lens. After injection, Polysporin ointment was applied to the puncture site. For the posterior approach, the needle was inserted through the sclera and retina at the time of ON transection; this route avoided direct injury to the iris or lens. All experiments to determine the range of effective times for BDNF and control injections, as

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Abbreviations: BDNF, brain-derived neurotrophic factor; CNS, central nervous system; diI, 1,1′-dioctadecyl-3-3-3′,3′-tetramethylindocarbocyanine perchlorate; HRP, horseradish peroxidase; ON, optic nerve; PN, peripheral nerve; RGC, retinal ganglion cell; SC, superior colliculus.

Table 1. RGC survival 2 weeks after ON transection: Effects of different amounts of BDNF

BDNF, μg	Fluorogold-labeled RGCs per mm², mean ± SD
0.0	305 ± 253 (n = 4)
0.5	574 ± 147 (n = 4)
2.5	907 ± 71* (n = 3)
5.0	814 ± 165* (n = 3)

One-way ANOVA ($P < 0.001$).
*Different from 0.0 BDNF, Bonferroni t test ($P < 0.05$).

well as the responses to multiple injections, were done by the anterior route to avoid repeated orbital dissections. In the eyes that had been injected via the anterior route, the lens was often opacified, particularly after multiple injections. With the posterior approach, the lens remained clear.

Chinese hamster ovary-derived human BDNF, provided by Regeneron Pharmaceuticals (Tarrytown, NY), was dissolved in 5 μl of a 1% solution of bovine serum albumin in phosphate-buffered saline (BSA/PBS). A dose–response analysis indicated that 2.5 and 5 μg of intraocular BDNF were equally effective in increasing RGC survival at 2 weeks but that 0.5 μg of BDNF was not significantly different from BSA/PBS alone (Table 1). Subsequently, each injection, made over ≈30 sec, consisted of 5 μg of BDNF or the same volume of BSA/PBS solution without BDNF. In other animals, the eye was punctured with a 26-gauge needle but no injection was made.

Retinal Areas. The flat-mounted retinas were drawn by camera lucida and their areas were measured with the aid of an Image-1 analysis system (Universal Imaging, West Chester, PA). Multiple injections by the anterior approach tended to cause shrinkage of the retinas. However, 2 weeks after ON transection, the areas of the experimental retinas were decreased by 5% or less compared to those of the uninjured, contralateral eye.

Examination of the Retinas. One to 8 weeks after ON transection, the animals were perfused with 4% paraformaldehyde. Both the left (ON lesion) and right (intact control) retinas were dissected, fixed for an additional 30 min, flat-mounted on glass slides, and examined by fluorescence microscopy (excitation filter, 355–425; barrier filter, LP 460) to determine the densities of surviving RGCs. To visualize RGC axons, additional experimental and control retinas were immunostained with RT97 (1), a monoclonal antibody that recognizes the 200-kDa neurofilament subunit.

RGC Densities. Densities were determined for experimental and control retinas by counting the number of Fluorogold- or HRP-labeled neurons in three standard areas of each retinal quadrant, for a total area of 0.965 mm² per retina (12). The results were analyzed with the SIGMASTAT program (Jandel, Corte Madera, CA), which first tests the data for normality and equal variance. Data that met the criteria for parametric tests were analyzed by a Student t test (paired groups) or by a one-way ANOVA and a subsequent Bonferroni t test (more than two groups). Groups of data that failed tests for normality and equal variance were analyzed by the nonparametric Kruskal–Wallis ANOVA followed by Dunn's test (14).

RESULTS

In uninjured control retinas, there were 2127 ± 444 Fluorogold-labeled RGCs per mm² (mean ± SD; n = 15). Such RGC counts persisted for at least 3 months (M. J. Berkelaar, G.M.B., and A.J.A., unpublished observations) and were similar to those observed with another fluorescent label, dil (11). The transection of the ON close to the eye caused

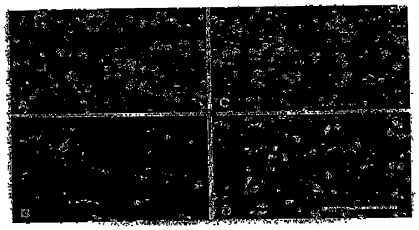

FIG. 1. Fluorogold labeling of RGCs in segments of a flat-mounted control retina (*A*) and in retinas 2 weeks after ON transection close to the eye (*B–D*). All photographs were taken 1 mm from the optic disc. (*A*) Intact retina. The perikarya and proximal dendrites of RGCs are delineated by punctate Fluorogold fluorescence that is most intense in the perinuclear cytoplasm. (*B*) ON cut without injections. There are few labeled RGCs; many of the labeled elongated cells are microglia that contain fluorescent material, presumably phagocytosed from degenerating RGCs. (*C*) Multiple BDNF injections (days 0, 3, and 7 after ON transection). The number of RGCs appears to be similar to the intact retina but there are occasional elongated microglial cells. (*D*) Multiple BSA/PBS injections. Fewer RGCs are apparent and there are more intensely fluorescent microglial cells than in the BDNF-treated retinas. (Bar = 100 μm.)

marked decreases in the numbers of Fluorogold-labeled RGCs in the uninjected eyes (Fig. 1). The RGC densities in these retinas were 1203 ± 149 cells per mm² (57% of controls) 7 days after axotomy, 484 ± 68 (23%) at 10 days, and 257 ± 74 (12%) at 14 days.

Increased Survival of Axotomized RGCs after BDNF or Control Injections. Two weeks after ON transection, more Fluorogold-labeled RGCs were apparent in the retinas from the animals that received the injections of BDNF or vehicle than in the untreated retinas (Fig. 1). The extent of the BDNF

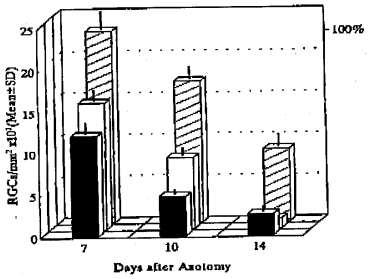

FIG. 2. Effects of BDNF and BSA/PBS injections on RGC survival after ON transection. One week after single posterior injections of BDNF into the vitreous chamber (hatched bars), RGC densities were similar to those of intact retinas (100%), while in the BSA/PBS-injected (open bars) and the uninjected (solid bars) retinas, RGC densities were 77% and 57% of controls, respectively. At 10 and 14 days after axotomy, RGC densities decreased in all groups but significantly more RGCs survived in the BDNF-injected eyes (one-way ANOVA; $P < 0.001$).

Table 2. RGC densities after ON transection: Effects of single (day 0) or repeated injections

Injection, route	Fluorogold-labeled RGCs per mm², mean ± SD			
	BDNF	BSA/PBS	Puncture	No injection
One-week survival*				
Single, posterior	2400 ± 207‡§ (n = 5; 113%)	1640 ± 153 (n = 4; 77%)	—	1203 ± 140 (n = 3; 57%)
Single, anterior	2445 ± 390§ (n = 3; 115%)	2171 ± 84§ (n = 3; 102%)	—	
Two-week survival: Single injection*				
Single, posterior	866 ± 163‡§ (n = 4; 41%)	121 ± 17 (n = 4; 6%)	—	257 ± 74 (n = 11; 12%)
Single, anterior	885 ± 153‡§ (n = 5; 42%)	426 ± 274 (n = 6; 20%)	612 ± 310 (n = 6; 29%)	
Two-week survival: Repeated injections†				
Repeated, anterior	1428 ± 255¶,a (n = 14; 67%)	1075 ± 123a (n = 8; 51%)	615 ± 402b (n = 3; 29%)	257 ± 74 (n = 11; 12%)
Four-week survival*				
Single, anterior	323 ± 156‡§ (n = 3; 15%)	60 ± 28 (n = 3; 3%)	245 ± 122 (n = 4; 12%)	64 ± 37 (n = 6; 3%)
Repeated, anterior	596 ± 85‡§‖,a (n = 4; 28%)	351 ± 42‖,a (n = 3; 17%)	246 ± 98b (n = 4; 12%)	

Percentages represent proportion of uninjured control (2127 ± 444 cells per mm²; n = 15). Statistical analyses by group: *, significant differences in means, one-way ANOVA ($P < 0.001$); †, significant differences in medians, Kruskal–Wallis one-way ANOVA on ranks ($P < 0.001$). Pairwise comparisons within groups, Bonferroni t test ($P < 0.05$): ‡, different from BSA/PBS; §, different from no injection; ¶, different from no injection, Dunn's test ($P < 0.05$); ‖, different from puncture.
[a]Injections on days 0, 3, 7, and 10.
[b]Punctures on days 0, 3, and 5.

effect was further documented by the counts of RGCs in standard retinal areas. While the RGC population declined to 57% 1 week after axotomy in the untreated retinas, the RGC counts remained normal after a single intraocular injection of BDNF by the posterior ocular route. At this time after injury, RGC numbers were 77% of normal when only the BSA/PBS vehicle was administered (Fig. 2). With single anterior injections on day 0 (Table 2), RGC densities at 7 days were normal with either BDNF or vehicle, suggesting that the injury associated with this route of injection initiated endogenous trophic responses that were more powerful than those triggered by the use of the posterior route.

During week 2 after axotomy, the numbers of surviving RGCs decreased for all groups (Fig. 2) but the values for the BDNF-treated retinas (41–42% of normal) remained significantly greater than for the vehicle-injected (6% after posterior injections; 20% with anterior injections) or uninjected retinas (12%) (Table 2). The different effects of vehicle injections by the anterior and posterior routes suggest that the trophic responses that are triggered by injury to anterior parts of the eye are more prolonged, as well as more intense, than those caused by posterior injections. The effects of single intraocular injections were approximately the same when given on day 0, 3, or 5; RGC densities at 2 weeks ranged from 42% to 47% of normal with BDNF and from 20% to 37% with vehicle compared to 12% in the untreated retinas (Table 3). By pooling the data for these 3 days, it was possible to show that the effect of BDNF injection was significantly greater than that of BSA/PBS (Table 3). With single injections on day 7 or 10, however, the number of RGCs declined to the range of the vehicle-injected or untreated retinas, presumably because they were administered after a large proportion of the injured RGCs had already died (Table 3) (33). The effects of day 0 injections of BDNF on RGC survival at 4 weeks were statistically significant (Table 2) although less marked than at 2 weeks.

When BDNF was injected 3 or 6 days before ON transection, RGC densities at 2 weeks were 37% and 34% of the densities of intact retinas and significantly greater than the 12% survival 2 weeks after ON cut without injections (one-way ANOVA; $P < 0.001$). This finding suggests that exposure of intact neurons to the neurotrophin helps them overcome subsequent injury.

Greater numbers of RGCs survived to 2 weeks with repeated anterior injections during week 1 after axotomy than with single injections (Table 2)—67% with BDNF and 51% with vehicle compared to 12% in the untreated retinas. These effects declined when the injections were discontinued. By 4 weeks, the numbers of surviving RGCs fell to 28% of normal for BDNF and to 17% for BSA/PBS, compared with 3% for the untreated injured retinas (Table 2). In another group of animals that received BDNF or BSA/PBS injections on days 0, 3, and 5 (data not shown), RGC densities for both groups were <200 cells per mm² (9% of normal) at 6 and 8 weeks. To determine whether some of the RGCs could be sustained for longer periods by more widely spaced injections, BDNF was injected weekly for 8 weeks. Although such retinas appeared to have greater numbers of RGCs at 4 and 6 weeks than in comparable retinas without injections, RGC densities could not be reliably counted because more than four injections caused shrinkages of 20–40% in the retinal areas.

After single or repeated eye punctures without injections, RGC numbers at 2 and 4 weeks approximated those obtained with single injections of vehicle (Tables 2 and 3). This finding

Table 3. RGC densities 2 weeks after ON transection: Effects of single injections at different times after axotomy

Anterior injection	Fluorogold-labeled RGCs per mm², mean ± SD			
	BDNF	BSA/PBS	Puncture	No injection
Day 0[b]	885 ± 153‡ (n = 5; 42%)	426 ± 274 (n = 6; 20%)	612 ± 310 (n = 6; 29%)	257 ± 146 (n = 11; 12%)
Day 3*	1007 ± 322‡ (n = 4; 47%)	528 ± 301 (n = 4; 25%)	495 ± 208 (n = 3; 23%)	
Day 5†	986 ± 206‡§ (n = 3; 46%)	781 ± 79§ (n = 3; 37%)	415 ± 317 (n = 5; 20%)	
Days 0, 3, and 5†	951 ± 219‡¶‖ (n = 12; 45%)	540 ± 276 (n = 13; 25%)	517 ± 288 (n = 14; 24%)	
Day 7	480 ± 42 (n = 2; 23%)	329 ± 54 (n = 2; 15%)	218 ± 234 (n = 4; 16%)	
Day 10	165 ± 45 (n = 4; 8%)	218 ± 79 (n = 4; 12%)	—	

Percentages represent proportion of uninjured control (2127 ± 444 cells per mm²; n = 15). Statistical analyses comparing injections on different days (by row): *, significant differences in medians, Kruskal–Wallis one-way ANOVA on ranks ($P < 0.01$); †, significant differences in means, one-way ANOVA ($P < 0.001$). Pairwise comparisons within groups (rows): ‡, different from no injection, Dunn's test ($P < 0.05$); §, different from no injection, Bonferroni t test ($P < 0.05$); ¶, different from puncture, Bonferroni t test ($P < 0.05$); ‖, different from BSA/PBS, Bonferroni t test ($P < 0.05$).

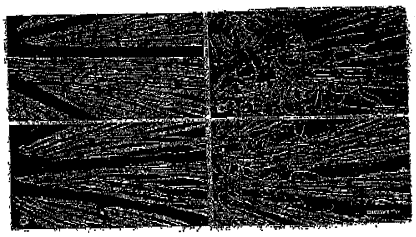

FIG. 3. RGC axons immunostained with an antibody (RT-97) that recognizes the phosphorylated 200-kDa neurofilament subunit. Retinal segments adjacent to the optic disc (on the left) from a flat-mounted control retina (*A*) and retinas 2 weeks after ON transection (*B–D*) are shown. (*A*) Intact retina. RT-97 immunoreactive axons course in bundles toward the optic disc. (*B*) ON cut without injections. Axon bundles are small and many degenerating fibers have a beaded appearance. No outgrowth is seen near the site of axotomy at the origin of the ON. (*C*) Multiple BDNF injections (days 0, 3, 7, and 10 after ON transection). Near the optic disc there is a profusion of axonal processes that overlap and extend in various directions. (*D*) Multiple BSA/PBS injections. Growth near the optic disc is less prominent than in the BDNF-treated retinas. (Bar = 100 μm.)

suggests that the endogenous trophic response had been triggered mainly by the puncturing of eye structures situated in the anterior portions of the eye and not solely by administration of the vehicle.

Injections Enhanced Axonal Regrowth Within the Eye but Failed to Stimulate RGC Axon Growth into the PN Grafts. In the eyes that received multiple anterior injections of BDNF or BSA/PBS, the retinas processed at 2 weeks for 200-kDa neurofilament immunoreactivity showed many newly formed axonal processes within ≈1 mm of the optic disc (Fig. 3). The location and appearance of these processes suggested that they arose from RGC axons near the origin of the severed ON.

The effects of increased RGC survival and intraretinal growth on the extension of RGC axons into PN grafts were investigated 6 weeks after ON transection and PN grafting (Table 4). As could be anticipated from previous studies (1), attachment of the PN graft further enhanced RGC survival: RGC densities with both PN grafts and BDNF injections were more than double those of BDNF alone or grafts alone. In spite of an ≈5-fold increase in the number of RGCs that survived in the BDNF-treated retinas, the number of RGCs that regenerated their axons into the PN grafts was similar for both the BDNF-treated and the nontreated groups—38 ± 25 and 28 ± 14, respectively.

Table 4. RGC survival and axon growth into PN grafts

	RGCs per mm², mean ± SD	
	BDNF†	No injection
Survival, no PN graft‡	196 ± 47* (n = 3)	7.5 ± 1 (n = 2)
Survival, with PN graft§	491 ± 71** (n = 6)	82 ± 36 (n = 5)
Regenerated¶	38 ± 25 (n = 6)	28 ± 14 (n = 5)

*, Different from no injection, Student's t test ($P < 0.05$); **, different from no injection, Student's t test ($P < 0.001$).
†Injections on days 0, 3, and 7.
‡Fluorogold-labeled RGCs.
§Fluorogold + HRP-labeled RGCs.
¶HRP-labeled RGCs.

DISCUSSION

BDNF Enhanced Survival of Injured RGCs. The greater survival of axotomized RGCs observed after intravitreal administration of BDNF is consistent with the hypothesis that this molecule is an important survival factor for these neurons. Using the posterior injection route that minimized the survival effects of control injections, virtually all RGCs were present 1 week after single injections of BDNF on day 0. This effect of BDNF contrasts with the loss of nearly one-half of the axotomized RGCs in the untreated retinas and approximately one-quarter of the RGCs after injections of BSA/PBS.

The early death of most of the RGCs axotomized near their cell bodies presumably reflects the loss of trophic support provided by both their targets and the nonneuronal components of the ON and tract. Their absence may render these injured nerve cells totally dependent on exogenous or intraocular sources of molecules required for survival. A strict dependency of axotomized CNS neurons on an exogenous supply of trophic factors was also a feature of experiments in which nerve growth factor was infused intraventricularly to prevent the loss of cholinergic nerve cells after transection of the fimbria fornix in rats (15–17). In such experiments, most of the rescued cells died soon after the neurotrophin was discontinued (18, 19).

The rapid loss of RGCs that occurred when single or multiple injections of BDNF were stopped may explain the lack of a significant survival effect reported by Mey and Thanos (20). At 3, 5, and 7 weeks after ON transection 5 mm from the eye and intraocular administration of BDNF, Mey and Thanos reported 2- to 3-fold increases in the numbers of axotomized RGCs but the differences were not statistically different from the effect of control injections.

Effects of Eye Injury. The enhanced survival of RGCs caused by intravitreal administration of the vehicle for BDNF (BSA/PBS) was greater and more prolonged when the eye was injured via an anterior approach than when the posterior route was used for the injections. Moreover, much of the trophic effect of vehicle injections could be reproduced by anterior eye punctures without injections into the vitreous chamber. Thus, it is likely that the effects of the control injections were largely due to injury of structures in the anterior part of the eye. The possibility that eye injury could trigger the release of trophic molecules from intraocular sources was also suggested by studies of photoreceptor cell lesions in rats. The loss of these cells due to a genetic defect or exposure to constant light was not only prevented by the injection of trophic molecules but also by control injections or needle insertion (21–23).

Recent studies in our laboratory indicate that the iris may be an important intraocular source of molecules that can support RGC survival. While it was previously demonstrated that the iris could release nerve growth factor (24), we have found that this structure also expresses BDNF and neurotrophin 4 mRNAs (T. N. Jelsma, S.M.-R., D.B.C., G.M.B., and A.J.A., unpublished observations). These neurotrophins could be produced in the iris by Schwann cells, which are known to release growth factors after injury (25, 26) or by muscle (27, 28). Thus, the injury-related effects on the survival of neurons in the retina may be due to the intraocular synthesis and release of molecules that are specific trophins for RGCs. By analogy with injury-induced responses in other parts of the CNS, RGC survival can also be influenced by changes in the expression of specific receptors (29) or by the synthesis and release of other trophic molecules known to be present in the eye such as acidic or basic fibroblast growth factors (30) and ciliary neurotrophic factor (31). Our finding that anterior ocular injections had a more prolonged survival effect than posterior injections suggests that the critical

molecules may be released from endogenous sources in a protracted fashion. Such an effect might be expected if Schwann cells or muscle in the injured iris were a source of these molecules.

RGC Survival and Axonal Regeneration. Injections of either BDNF or BSA/PBS caused a striking proliferation of RGC axons near the optic disc. This finding may indicate that conditions created by administration of BDNF or by release of this and other molecules within the eye itself may not only stimulate RGC survival but locally enhance branching and growth from the stump of most of the interrupted RGC axons.

It is unclear, however, why the 5-fold increase in the number of surviving RGCs and the abundant local regrowth of RGC branches observed around the optic disc of the treated retinas were not associated with a significant increase in the number of RGC axons that regenerated into the PN grafts attached to the ON. It is possible that BDNF and other trophic substances released within the eye may be important for the survival of RGCs but that additional molecules may be required to stimulate the lengthy extension of the axons into the grafts. Conceivably, concentration gradients created by intraocular injections of BDNF may have induced the RGC axons to grow into the eye rather than into the grafts. Evidence of a role for gradients of growth factors on the guidance of growth cones has been provided for nerve growth factor by *in vitro* and *in vivo* experiments (32). Finally, it remains possible that only a particular subset of RGCs is able to regrow their axons into peripheral nerve grafts or that the narrow glial interface that is left between the eye and these grafts is a barrier that can be overcome by only a few RGC axons.

While the present studies indicate that it may indeed be possible to prevent the loss of significant numbers of injured RGC neurons by the timely provision of specific trophic molecules from endogenous or exogenous sources, some of the caveats raised by these investigations will need to be considered in the design of future experiments aimed at promoting the regeneration of injured CNS neurons. Our studies have now focused on the development of safe methods for sustained intraocular delivery of BDNF and other critical molecules and on the understanding of the conditions that divert the extension of RGC axons into PN grafts "bridging" the eye and the SC of these animals.

The assistance of M. David, J. Laganière, J. Trecarten, and W. Wilcox is gratefully acknowledged. We thank Dr. M. Rasminsky for assistance with the data analyses. This work was supported by grants from the Multiple Sclerosis Society of Canada, The Medical Research Council, and the Canadian Network on Neural Regeneration.

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